GENETIC RELATIONSHIPS AMONG

Spiranthes parksii AND CONGENERIC SPECIES

A Thesis

by

CATHERINE WALTERS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Botany

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Approved by:

Chair of Committee,	James Manhart
Committee members,	Alan Pepper
	Hugh Wilson
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ABSTRACT

Genetic Relationships Among

Spiranthes parksii and Congeneric Species (December 2005)

Catherine Walters, B.S., University of Kansas

Chair of Advisory Committee: Dr. James Manhart

Using four AFLP markers and seven polymorphic microsatellite loci, we examined the genetic structure of the rare and endangered *Spiranthes parksii* Correll (Orchidaceae). *Spiranthes parksii* is not distinguishable from sympatric *S. cernua* (L.) Rich based on these data, though low levels of polymorphisms exist within both. These low levels of genetic diversity are likely a result of high levels of agamospermic reproduction through adventitious embryony. These results suggest that both *S. parksii,* as well as the sympatric, open flower form of *S. cernua,* are products of the more widely distributed *S. cernua* complex. Further, another local form of *S. cernua*, distinguished by its tendency to produce closed flowers is genetically distinct from both *S. parksii,* as well as the open-flower form of *S. cernua,* as shown by AFLPs and microsatellite loci. This is the first known set of microsatellite primers developed specifically for use in *Spiranthes*. The application of these markers may be used to address other unresolved relationships among species of *Spiranthes*, many of which are also endangered or have populations in decline.

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I would like to thank all of the following people for their enthusiasm for this project and their support for me personally as well as professionally. I am indebted to Drs. Manhart and Pepper for their support and commitment to me and for their time and mentorship. I thank the *Spiranthes* recovery team for informative discussions and particularly Dr. Fred Smeins, Kathy Parker and James Thomas for their many hours of aid in the field with finding plants. I am grateful to Lucy Dueck for her insight and knowledge of *Spiranthes* from the eastern US as well as her willingness to share DNA samples. I would like to express my gratitude to Karen Harris for her guidance with the AFLP protocol, and to Will Shaw for help with GIS mapping of populations. Last, this work was funded by a Section 6 grant from USFWS.

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CHAPTER I

INTRODUCTION

Spiranthes is one of a few orchid genera with a cosmopolitan distribution and its members are characterized as a taxonomically difficult group due to substantial variation in traditionally important characters such as plant size and floral morphology as well as reproductive biology. Depending on the treatment, a few dozen to three hundred species are delimited (Correll 1950). All members are terrestrial and have an inflorescence with flowers arranged in a single to several-ranked spiral around a central axis. Leaves are arranged in a basal rosette and are present during spring in both *S. parksii* Correll and *S. cernua* (L.) Rich, at which time it is impossible to distinguish the two species, but often absent during their fall bloom period. Even when in flower, *S. parksii*, is easily confused with more widely distributed species, particularly *S. cernua*, which is sympatric throughout *S. parksii's* range. *Spiranthes parksii* was first described (Correll 1950) based on its unique morphology including smaller size, lateral sepal shape and position, and more open spiral of the inflorescence. Its upcurved and long lateral sepals relative to the petals as well as its white-tipped floral bracts most easily distinguish *S. parksii* from *S. cernua*.

Spiranthes parksii, also known as Navasota Ladies' Tresses, is an endemic of the post oak savanna region of east-central Texas. It was first discovered along the Navasota

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This thesis follows the style of *Molecular Ecology.*

River in 1945 and was named for its collector, H.B. Parks. Many years passed without sightings, prompting the Smithsonian institute to declare it extinct in 1975. However, in October of 1978, twenty plants were located northwest of Navasota, TX (Catling & McIntosh 1979). This species was listed on May 6, 1982 as an endangered species under the Endangered Species Act of 1973 due to low numbers of plants observed during its fall blooming season as well as its more specific habitat requirements. The primary threats to this species include habitat loss due to urban development, secondary succession and possibly herbivory.

Morphological, cytogenetic and reproductive studies have centered on the closely related and broadly distributed *Spiranthes cernua* (L.) Rich (Sheviak 1982, Schmidt 1992, Catling 1982 & 1983), yet the genetics, population dynamics and ecology of *S. parksii* remain poorly understood. Major goals of this study were to 1) examine the genetic structure of both *S. parksii* and *S. cernua* using chloroplast sequence data, AFLPs and microsatellite loci, 2) determine the reproductive mode and define the breeding system for *S. parksii*, and 3) determine the evolutionary origins of *S. parksii.*

Habitat and Distribution of *S. parksii*

Most known populations are centered along a narrow band in Brazos and Grimes counties near the Navasota River and can often be found in open wooded areas near small, upland streams and drainages (Fig. 1). Additional outlying plants were found in the Angelina National Forest, approximately 100 miles east of the bulk of the

distribution (Bridges & Orzell 1989), as well as in Limestone County (2004) and in Bastrop County at the Stengl Biological Research Station (2004).

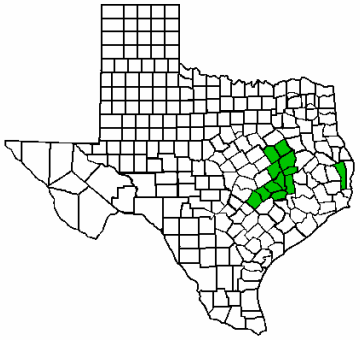

Fig. 1 Distribution of known populations of *Spiranthes parksii*. Populations are known for Brazos, Grimes, Burleson, Washington, Madison, Leon, Robertson, Limestone, Freestone, Bastrop, Lee and Jasper counties. (Texas A&M Bioinformatics Working Group).

Species Descriptions

Spiranthes parksii is commonly known as Navasota Ladies' Tresses and has distinguishing morphology characterized by a truncated labellum, up-curved lateral sepals, and flowers ascending to perpendicular to the axis of the inflorescence rather than perpendicular or nodding as in the open flower form of *S. cernua*. Additional supporting morphological characters include more pubescence on the bracts and flowers and a white-tipped bract typically curved away from the flower (Fig.2). Chromosome counts for this species are 2n=4X=60 indicating it is a tetraploid (C. Sheviak, unpublished report).

Fig. 2 Images of *Spiranthes parksii.* Flowers shown have a white tipped bract, a truncated lip, and lateral sepals which curve upwards and are longer than the petals.

Spiranthes cernua is a highly polymorphic compilospecies which occurs in many geographically variable forms. Chromosome counts indicate that most individuals are tetraploid (2n=4X=60), though numerous aneuploids (2n=4x=60 ± 2) as well as triploids (2n=3x=45) are known to occur. In east-central Texas, there are two general forms described by Sheviak, which are discussed below.

The woodland form has open flowers and an expanded labellum which is as long as the lateral sepals. It was described based on its occurrence in more shaded areas in a woodland habitat. The orientation of single flowers ranges from perpendicular to the axis of inflorescence or nodding, but is never ascending. Additional characters, which are less pronounced to varying degrees, include a brighter, whiter color of the petals and sepals and a lesser degree of pubescence on floral structures compared to *S. parksii*. Floral

bracts are green-tipped and typically curve in towards the flower (Fig. 3). Here, this form will be referred to as *S. cernua* (of).

The second form is the peloric form, which was described based on the labellum's non-distinct appearance as a third petal. It is also referred to as cleistapogamous form for its suspected method of reproduction as well as the southern prairie race for its association with more open prairie areas of the southern U.S. Here this form will be referred to as *S. cernua* (cf) to avoid assumptions about breeding system or habitat. For this study, this form was identified based on the presence of closed, ascending flowers which are greenish-white to white (Fig. 4). However, plants may occasionally produce several open flowers on an inflorescence or produce an inflorescence with entirely open flowers (Fig. 5). The degree that flowers open is reportedly temperature dependent, with flowers closed in warmer temperatures and open in cooler temperatures. Mature plants of this form often produce plump ovaries on all of the flowers of the inflorescence, which suggests either agamospermic or autogamic reproduction occurs in these individuals.

Fig. 3 Images of *Spiranthes cernua* open flower form. Flowers are white with green-tipped bracts and lateral sepals are as long as petals.

Fig. 4 Images of *Spiranthes cernua* closed flower form. Flowers shown are closed while seeds are developing inside the ovary.

Fig. 5 Images of *Spiranthes cernua* closed flower form with flower slightly open.

Reproductive Biology

The breeding systems of *Spiranthes* are quite variable and include agamospermy (through adventitious embryony), autogamy, outcrossing and mixed-breeding systems involving various levels and combinations of the above (Catling 1982 & 1983). Unlike many orchid species, *Spiranthes* do not have species-specific pollinators. *Bombus* species and solitary leaf-cutting bees of the Megachilidae are reported to be the most common pollinators for most species of this genus. Though pollinia have on rare occasion been observed on petals and sepals of *S. parksii* flowers, indicating some level of insect activity, the pollinators for the species are unknown. Small flower size in *S. parksii* may prevent bumblebees from accessing pollinia. Bumblebees have been reported on *S. parksii* (H. Wilson, unpublished report) however, it is not clear whether they serve as effective pollinators. Protandry and early development of flowers positioned lower on the spike are thought to promote outcrossing in the genus, however, many species of *Spiranthes* deviate from this mode of reproduction.

Polyembryony has long been recognized in *Spiranthes* (Leavitt 1901; Swamy 1948) and was further associated with a form of agamospermy known as adventitious embryony in which embryos are formed mitotically, without pollination, from the inner integument rather than an unreduced egg cell. This mode of reproduction resembles vegetative reproduction in that all offspring produced carry the maternal genotype. Agamospermy has been considered to be an adaptation in pollinator-limited populations as it may be more common among more peripheral individuals of a population in *S. cernua*. (Schmidt 1992). Polyembryonic seeds (Fig. 6) typically contain between two and six embryos, yet adventitious embryony may not exclude the possibility of sexually produced embryos within the same seed. While *S. cernua* exists in sexual, agamospermic and mixed races (Sheviak 1982; Catling 1982), the mode of reproduction in *S. parksii* is not defined, though polyembryonic seeds are reportedly as high as 80-90% (Catling & McIntosh 1979). There is evidence for apomixis for this species; however, the frequency of this mode of reproduction is unknown.

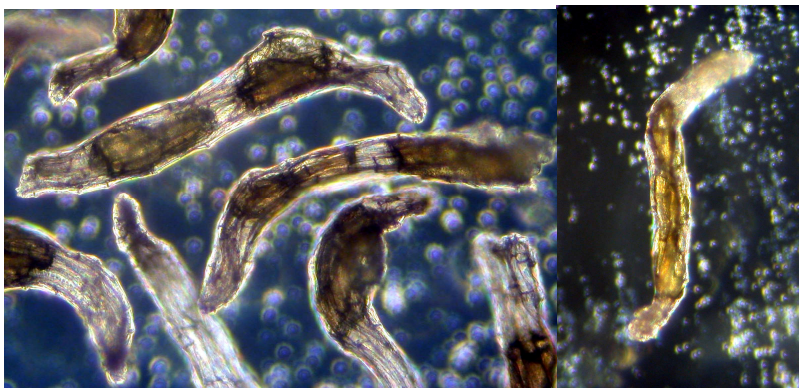

Fig. 6 Polyembryonic seeds of *Spiranthes cernua* closed flower form.

Though the focal species of this study is *S. parksii*, other closely related species were incorporated, providing a framework for comparative biology of this species. These species include *S. cernua*, *S. magnicamporum* Sheviak as well as *S. vernalis* Engelm. & Gray. While *S. cernua* is suspected to exist in outcrossing, agamospermic and mixed races, *S. magnicamporum*, a diploid relative of and one of the putative progenitor of *S. cernua*, is thought to be mostly outcrossing with other diploid relatives. *Spiranthes vernalis*, though not associated with the *S. cernua* complex, is potentially useful for comparison due to the fact it is an obligate outcrossing species in which agamospermy is not known to occur. In this species, seeds are regular in shape and either lacking an embryo entirely, indicating failed fertilization, or contain a single embryo positioned centrally within the seed (Fig. 7).

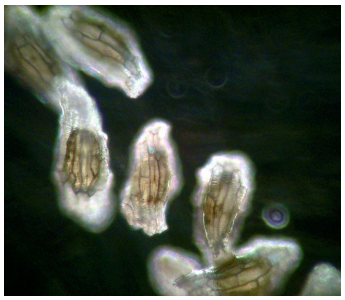

Fig. 7 Monoembryonic seeds of *Spiranthes vernalis*.

Population Sampling and DNA Extraction

Permit number SPR-1202-258 issued from Texas Parks and Wildlife to collect *S. parksii* on state properties is valid through Dec. 4, 2005. Voucher specimens for each species

were collected and banked at the TAMU Herbarium. Populations of *S. parksii* as well as local *S. cernua* were sampled and GPS coordinates for each specimen were recorded (Fig. 8).

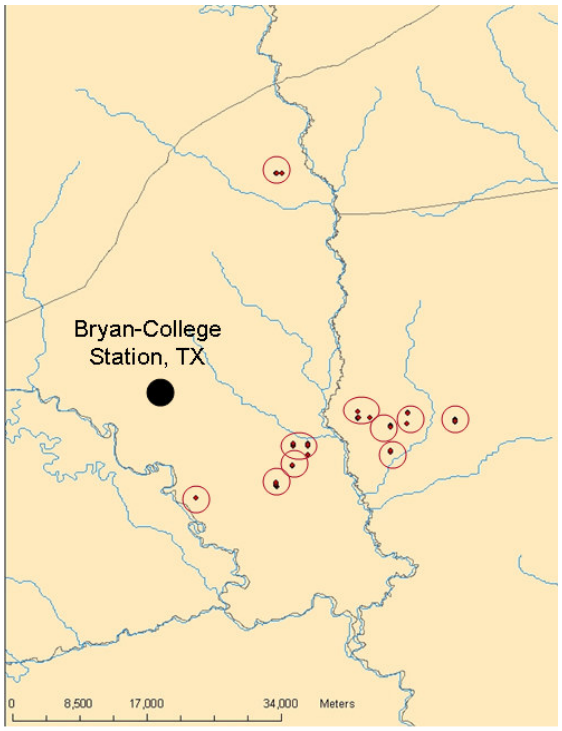

Fig. 8 Distribution of core populations of *Spiranthes parksii*. Plants were sampled from ten populations indicated on the map in Brazos and Grimes counties. Additional samples were obtained from Limestone and Bastrop counties which are not indicated on this map.

A simple field DNA extraction method was used that has produced genomic DNA of suitable quality and quantity for PCR-based applications (Pepper & Norwood 2001). Sampling was nondestructive and genomic DNA was obtained from tissue of only one or two cauline or floral bracts. DNA was ground in an extraction buffer

(200mM Tris pH 8.0, 250 mM NaCl, 25mM EDTA pH 8, .5% SDS) until the extraction was completed by a series of isopropanol precipitations in the lab. The final pellet was resuspended in 100ul .5X TE buffer and stored at -20º. DNA was extracted from 296 individuals of *S. parksii* as well as 209 individuals of *S. cernua* from ten populations in the core distribution in Brazos and Grimes counties, Texas during fall 2003. DNA was obtained from 19 plants identified as *S. parksii* and one as *S. cernua* (cf) in Limestone county fall 2004. Three plants identified as *S. parksii*, 3 as *S. cernua* (cf) and four as *S. cernua* (of) form and one non-resupinate individual of *S. cernua* were sampled at the Stengl Lost Pines Biological Station in Bastrop County in fall of 2004. DNA from a population of *S. magnicamporum* was collected in Blanco County, TX in fall of 2003. DNA from populations of *S. vernalis, S. lacera* var. gracilis (Raf.) Raf. var. *gracilis* (Bigelow) Luer, and *S. praecox* (Walt.) S. Wats were also obtained by the same procedure.

CHAPTER II

CHLOROPLAST VARIATION

Introduction

Plastid DNA, including cpDNA, has been used in molecular systematics at various taxonomic levels. Several "universal primers" have been designed and tested on various taxa to determine which regions are most appropriate for studies at a given taxonomic level (Taberlet *et al.* 1991). The heavily used trnT-trnF region has been used within the Orchidaceae at the intraspecific (Trapnell *et al.* 2004), specific (Bellstedt *et al.* 2001), as well tribal levels (Salazar *et al.* 2003). Its utility in *Spiranthes* and more specifically within the *S. parksii* and *S. cernua* is unknown.

Primers designed from the more conserved gene regions are indicated by a black box, whereas the non-coding regions amplified by these primers are indicated by a black line (Fig. 9). These primers were tested on *Spiranthes* in attempt to find regions most suitable for use in *S. parksii*.

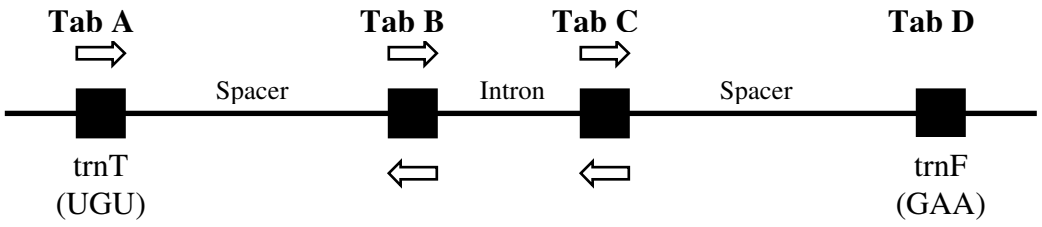

Fig. 9 TrnT-trnF spacer and intron regions of the chloroplast genome.

Materials and Methods

Sequencing

The chloroplast trnT-trnF non-coding region was PCR amplified with taq polymerase using previously designed primers (Taberlet *et al.* 1991; Cronn 2002) (Table 1). PCR thermal cycling for amplifications included an initial 2 min at 94, followed by 35 cycles of 1 min at 94C, 1 min at 50C, and 1 min at 72C, with a final extension period of 72C for 5 min. Amplified DNA was purified using sephadex spin columns, dried and resuspended in 20uL 0 .1mM Tris and sequenced using dye terminator cycle sequencing and the same primers for amplification were used for sequencing. Thermal cycling for sequencing included 96C for 2 min, followed by 60 cycles of 96C for 30 sec, 45C for 15 sec, and 60C for 4 min. Sequence reactions were purified using sephadex spin columns and then electrophoresed using an Applied Biosystems model 3100 genetic analyzer.

Table 1 cpDNA primer name and sequence. Primer name and sequence used for amplification and sequencing of trnT-trnF cpDNA region.

Primer Name	Sequence 5'-3'
trnT-F (tabA)	CATTACAAATGCGATGCTCT
trnT-F (alt Tab A)	CAAATGCGATGCTCTAACCT
trnT-F (alt Tab A interior)	AATATTACTGACTCCMTTTTKATTTTCKAG
trnT-F (TabB)	TCTACCGATTTCGCCATATC
trnT-F (Tab C)	CGAAATCGGTAGACGCTACG
trnT-F (Tab D)	GGGGATAGAGGGACTTGAAC
trnT-F (Tab E)	GGTTCAAGTCCCTCTATCCC
trnT-F (Tab F)	ATTTGAACTGGTGACACGAG

Results

Tab A&B

Amplification of Tab A was problematic in *Spiranthes*. This is not so surprising given that others using these same primers had amplification problems in various other taxa (Taberlet *et al.* 1991). This region amplified with a weak product and sequencing efforts were not completely successful. However, approximately 400 bp of readable sequence among 5 individuals *S. parksii*, 3 of *S. cernua* (of) and one *S. cernua* (cf) was obtained for this region using the alternate primer, TrnA2 for amplification as well as for sequencing reactions. While *S. cernua* (of) was identical to *S. parksii* in all samples, *S. cernua* (cf) differed by a one base insertion of the base thymine.

Tab C&D

No nucleotide variation was detected in a 570bp region among 5 samples of *S. cernua* (of), 3 of *S. parksii* and 1 of *S. cernua* (cf). However, several indels as well as base substitutions were present between these samples and *Spiranthes lacera* var. gracilis.

Tab E&F

Four samples of *S. parksii*, two of *S. cernua* (of), and one of *S. cernua* (cf) were sequenced at the Tab E&F intergenic spacer region. The size of this segment was approximately 502bp for all seven samples, however, 392bp of readable sequence across all samples was obtained at which all samples were identical.

Discussion

The chloroplast regions sequenced show no variation between samples of *S. cernua* (of) and *S. parksii*. A one base pair insertion detected in the 1,362 base pairs examined in *S. cernua* (cf) may be indicative of some differentiation of this form compared to *S. cernua* (of) or *S. parksii*. This additional base pair was previously dismissed as a sequencing error, though an independent attempt to sequence the same sample for the same region in another lab produced the same results (L. Dueck, personal communication). Though few samples were examined at these chloroplast regions, they appear to be invariant in the local members of the *S. cernua* as well as *S. parksii* and would not be informative in resolving relationships between the two. Chloroplast sequencing techniques are routinely used to address systematic questions at the specific level, but due to their slower mutation rates, they are not as informative at the population level or among closely related species in comparison to other techniques. The trnL intron region appears to be most informative of these regions examined for more distantly related species of *Spiranthes*, but uninformative for resolving relationships between *S. parksii* and *S. cernua*, thus no further efforts to sequence cpDNA segments were made.

CHAPTER III

AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

Introduction

AFLPs (amplified fragment length polymorphisms) are dominant markers used to examine DNA fragments which have been selectively amplified from a digested genome. Variation among individuals in fragments is created when a mutation occurs in the DNA region recognized by the enzyme used to digest the genome or by insertions or deletions in the amplification product itself. These mutations give fragments different mobility when run on a gel. Markers are dominant and scored in terms of their presence or absence in a particular individual. Bands of similar mobility are assumed to be identical by descent though this may not be the case as fragments of the same size may be generated from non-homologous regions of the genome. However, this type of homoplasy is considered to be negligible with this technique. In *Spiranthes romanzoffiana* Cham. from the British Isles, 86 polymorphic AFLP markers were detected, within and among populations, using three primer combinations across 205 samples (Forrest *et. al.* 2004). This technique is powerful in its ability to detect overall similarity or differences rapidly relative to other molecular techniques as no prior sequence knowledge of the species of interest is required. This feature makes AFLPs particularly useful in the study of non-model organisms, which are often understudied and poorly understood.

Materials and Methods

Following a modified protocol (Vos *et al.* 1995), approximately 100ng of genomic DNA quantified by a spectrophotometer was digested with a six base cutter, EcoRI, and a four base cutter, MseI, in a 37C water bath for two hours. Adapter primers were ligated to the digested DNA overnight in a 37C water bath. Adapter primer sequence is as follows: E-adapter 1= CTC GTA GAC TGC GTA CC. E-adapter 2= AAT TGG TAC GCA GTC TAC. M-adapter 1= GAC GAT GAG TCC TGA G M-adapter 2= TAC TCA GGA CTC AT. During the pre-selective round of amplification, an M+C primer sequence ACG ATG AGT CCT GAG TAA C was used in combination with a non selective E+O primer sequence GTA GAC TGC GTA CCA ATT C. During the selective amplification, four primer combinations (Table 2) were used on a set of 50 samples from 10 populations. The E-selective primer for each combination was labeled with and infrared dye for detection on either the 700 or 800 channel on the LI-COR 4200 DNA Analyzer.

Table 2 AFLP primer combinations. Primer combinations correspond to the IR channel on which they were run.

IR 700	**IR800**
E-GAA/ M-CAT	E-GGA/ M-CAT
E-CAA/ M-CAT	E-TGA/ M-CAT

5ul of each amplification product were pooled with 2ul of loading buffer and denatured at 95C for 2.5 min. DNA fragments were separated on an 8.0% denaturing

polyacrylamide gel (8.0% Long Ranger acrylamide[Cambrex, Rockland], 1.2X TBE, and 7M urea). To 30 ml of filtered gel solution, 200 µl of 10% ammonium persulfate and 20 ul of TEMED were added, and gels were cast using a LI-COR gel assembly (LI-COR). Gels were run at a constant wattage (40 W) for 3 h with a 1X TBE running buffer. AFLPs were scored manually.

Results

Four polymorphic markers were found in the *S. cernua* complex with three primer combinations, E-CAA/M-CAT, E-GAA/M-CAT and E-GGA/MCAT. The fourth primer combination used, E-TGA/ M-CAT, produced no polymorphic markers. Three markers scored were present only in the closed flower form of *S. cernua*. The fourth marker was present only in *S. parksii* and the open flower form of *S. cernua*. In marker 3, two samples of the closed flower form of *S. cernua* did not have bands found in other samples of the same morphology. Data were scored at polymorphic markers as presence or absence of bands. Individuals that contain a given marker were scored with a "1" while those without were scored with a "0". A neighbor joining tree was constructed in PAUP 4.02b based on average genetic distances between pair wise samples and a bootstrap analysis was performed with 1000 replicates (Fig. 10).

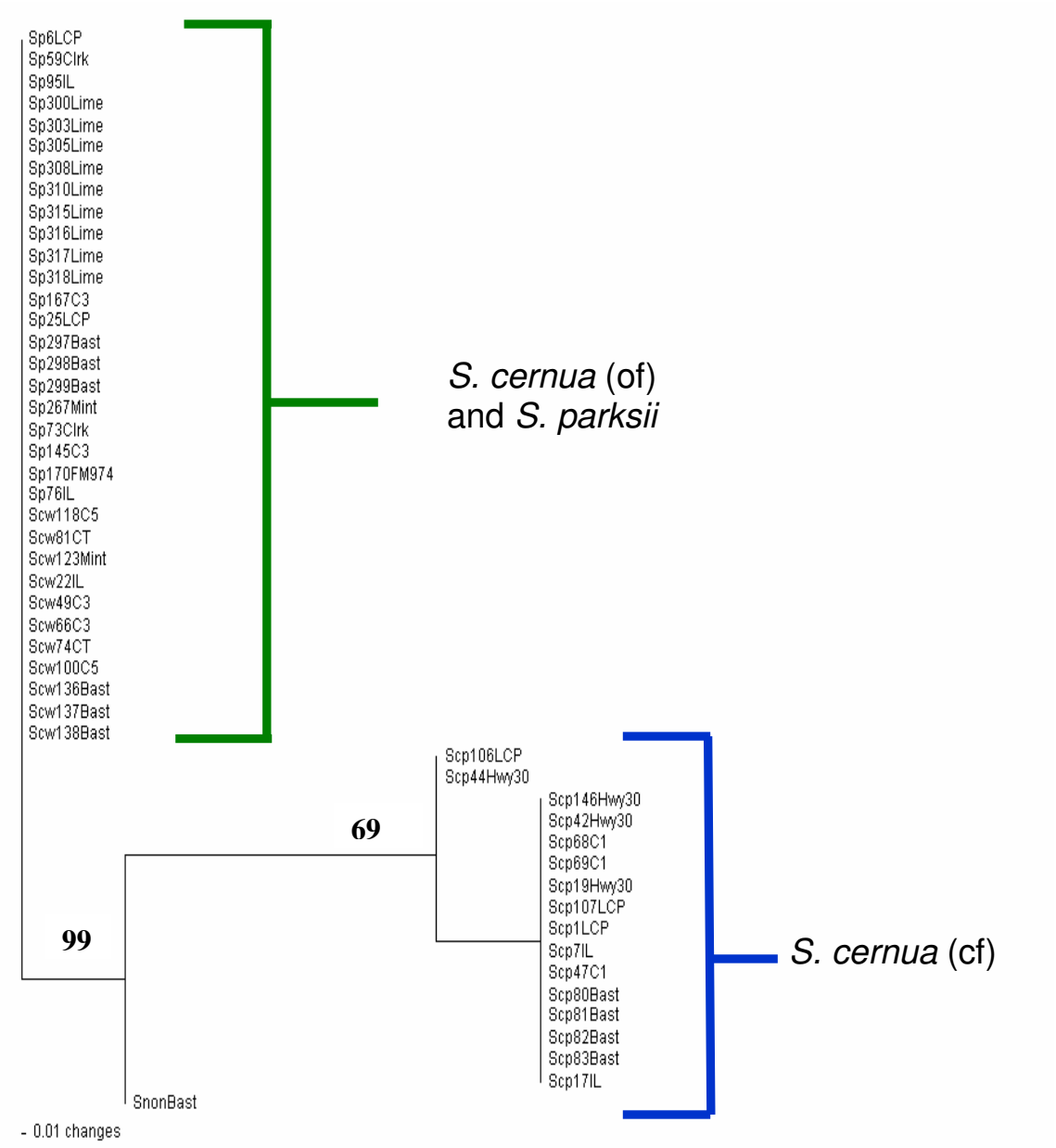

Fig. 10 NJ phylogram constructed from AFLP data. Samples labeled "sp" followed by a number represent *S. parksii* from 10 populations whereas samples labeled "scw" followed by a number represent *S. cernua* (of) from 6 populations. Samples labeled "scp" represent *S. cernua* (cf) from 5 populations. "SnonBast" represents a non-resupinate form. Population designations follow the sample ID. Percent bootstrap support values are indicated in bold.

Discussion

The resulting tree (Fig. 10) clearly shows a separation of the closed flower form from the open flower form of *S. cernua* as well as *S. parksii* with bootstrap support of 99% (Fig. 10). These results were surprising and seemed contrary to Sheviak's observation that floral form of *S. cernua* is an expression of environmental influences. The two forms of *S. cernua* were expected to cluster together and form a separate group from *S. parksii*. However, these results suggest that rather than forms, these are two genetically differentiated groups.

Generally, markers were either present in this form and absent *S. parksii* or *S. cernua* (of), though one marker was present in both *S. parksii* and *S. cernua* (of), yet absent in *S. cernua* (cf). Because none of the four polymorphic markers found were shared among the two, these results are strongly suggestive that the closed flower form of *S. cernua* is more distant from S. cernua (of) than previously thought. Two samples, scp44 and scp106, were more similar to *S. cernua* (of) or *S. parksii* because they did not contain one marker that was present in the other individuals of the same form. AFLPs based on the primer combinations used detected slight variation in this form, and more markers may detect more differences among them.

Further, *S. parksii* and *S. cernua* (of) are genetically indistinguishable from each other and contain no polymorphisms within these the four primer combinations used in the analysis. This lack of variation could be due to severe inbreeding, a common phenomenon in small populations which often results in an excess of homozygotes. Alternatively, low genetic variation could result from an apomictic reproductive mode,

in which case at least some loci would likely show fixed heterozygous patterns. A co-dominant marker, such as microsatellites, is needed to resolve this issue.

AFLPs are well-suited for finding levels of variation and overall similarity between samples as well as identifying clones and even potentially hybrids. However, they are limited in that they are dominant markers and do not contain allelic data or information regarding heterozygosity, both of which are critical components for examining breeding systems as well as conducting informed population management practices. Still, the high numbers of polymorphic markers, which can be obtained with relative speed and ease, help compensate for its limitations.

CHAPTER IV

MICROSATELLITE VARIATION

Introduction

Microsatellites are highly repetitive regions of the genome found in all eukaryotic organisms and are characterized by a base motif of two to six nucleotides in tandem repeats of up to 100 times. They are assumed to be evenly distributed over genomes (Dietrich *et al.* 1996), but rare within coding regions (Wang *et al.* 1994). Mutations at microsatellite loci occur primarily due to slip strand mis-pairing during DNA replication, which results in an increase or decrease in repeat length. Thus, variation is scored in terms of length of the number of repeats at a locus. These markers have several advantages over others in that variation has been found at microsatellite loci in populations with low levels of isozyme variation, they are locus specific (as opposed to RAPDs and AFLPs), are PCR based and require small amounts of tissue for amplification. Additionally, alleles can be unambiguously sized on a variety of capillary electrophoresis instruments that are becoming more widely accessible. Due to their co-dominance, hypervariability and selective neutrality, microsatellites are increasingly becoming the tool of choice in population genetic studies.

Molecular markers have been particularly useful in resolving relationships among closely related taxa particularly among difficult and morphologically variable groups (Avise 1994). The use of molecular genetics in population biology dates back to the 1960s when protein electrophoresis revealed extensive variation at alleles for allozymes (Lewontin & Hubby 1966; Harris 1966). However, protein electrophoresis is

thought to underestimate variation due to both undetectable differences in amino acids at the 3rd codon position as well as the slowly evolving nature of proteins. Previous analysis of isozymes detected no patterns of genetic partitioning in populations of *S. parksii* and *S. cernua* (H. Wilson, unpublished data). With highly variable loci such as microsatellites, the potential to resolve differences between closely related groups is much higher. This powerful tool is used not only to survey genetic variation, but also to investigate historical patterns such as detection of previous population expansions or contractions and bottlenecks. These markers are potentially useful in *Spiranthes* in addressing whether low genetic variation among individuals detected by AFLPs is due to inbreeding or apomixis.

Materials and Methods

A genomic library enriched for five microsatellite repeat sequences (TA_n, CA_n, GA_n, AGA_n, TGA_n, and ACA_n) was constructed for *S. parksii* using a highly modified and simplified protocol based on biotinylated-oligonucleotide capture methods (Kijas *et al*. 1994 and Prochazka *et al.*1996), with some of the modifications (Connell *et al.* 1998). BigDye terminator cycle sequencing (Perkin-Elmer Applied Biosystems) and vector-specific M13, T7 or TopF-2 forward and reverse primers were used to sequence clones. 336 clones were sequenced and screened for microsatellite regions. Primers developed for microsatellite loci were 18-25 bp in length with 40-60% GC content and a salt adjusted melting temperature of approximately 60C. Oligonucleotides were checked to minimize self-annealing and hairpin formation.

Primers were designed for 64 microsatellite loci of which 22 amplified on an initial test of 2-8 samples. Twelve of these were selected to screen for polymorphisms in a set of 48 samples including *S. parksii* and both forms of *S. cernua*. Amplification products were run on a high resolution agarose gel composed of 2% Metaphor (Cambrex, Rockland) and 2% Agarose I (Amresco, Solon) to screen for polymorphisms. No polymorphisms were detected by these means between *S. cernua* (of) and *S. parksii*. However, products of many *S. cernua* (cf) migrated differently on the gel. A set of twelve primers were selected to be fluorescently dye labeled and used with the Applied Biosystems (ABI) PRISM® 3100 genetic analyzer, an unambiguous method for sizing DNA fragments.

Microsatellite loci were PCR amplified with taq polymerase using either FAM, HEX or TET dye labeled primers (Table 3) in separate 20ul reactions using primers at a final concentration of .3uM for each forward and reverse primer. PCR thermal cycling included an initial 2 min at 94C, followed by 28-35 cycles of 1 min at 94C, 1 min at 55C, and 1 min at 72C, with a final extension period of 72C for 10 min. Two loci, 8A and 1A2, did not amplify consistently with dye labeled primers and were subsequently excluded from further analysis. Amplification products were prepared for capillary electrophoresis on the Applied Biosystems model 3100 genetic analyzer as follows. Products were diluted 1:20-1:60 depending on amplification strength, and then pooled with 9ul Hi-Di formamide and .15ul Rox dye standard for a final concentration of approximately .03 %. Amplification products were assigned to allele classes with GENESCAN and GENOTYPER software (Applied Biosystems).

Table 3 Characterization of microsatellite loci. Locus name is listed with the fluorescent dye used in parentheses. Forward and reverse sequences are listed 5'-3', followed by repeat motif and approximate amplicon size.

Locus	Primer sequence	Repeat Motif	Amplicon Size
3A (FAM)	F- AATGACTGATGACAGTCGAAG R- GCTATGCCATCACTCACGTG	$(CTT)_7$	64
8D2 (HEX)	F- CATTATCGTCGGTCACCGTT R-CATGAGCCTAGCGCGATCTT	$(CT)_{14}$	92
sp2-11H (HEX)	F AAACTAGGGTCAATAAAACTCCG R-ACATTAGGATGAATATTCTTTGGC	$(GA)_{25}$	222
sp2-12C (FAM)	F- TCTCCGTAGATATCACACTCG R- CAATAGTGCAACTTTGATGTCCG	$(GA)_{14}(A)_7$	139
sp3-9A (FAM)	F-AAGATATTTAAGATGCACAATCGC R-AACCCTTGTAGGATTTCATTGG	$(CT)_{25}(GA)(A)$	192
sp3-11B (TET)	F- TTCTCTCAATCCCATAGCTGG R- TTCTCGGTGTTCATCATCTCG	(CT) (CTT)	305
sp3-3A (HEX)	F- AT GCA ATA CAT AGC AGC CGC R- GCTGTATAAGATTTCGGTCTCC	$(GA)_9(GAA)_2$ $(GA)_3$	261
sp4-9C (Hex)	F- AGAATGTCCACAGTAACAGCG R- TTCATAAATAGCAGTTGTTCCAG	$(CT)_{18}(T)_9$	173
sp4-1A (FAM)	F- TGGGAATGACTCATCACAGTCG R- TGCCACGCCATCACTCACG	$(GAA)_9$	71
sp4-5E (TET)	F- AGGTGGAATTCTGTCACACTG R- CAACTCGAACTGATCTTCTGG	$(GAA)_7$	97

Results

A set of 96 samples were amplified with primers for 10 loci. Of the nine loci which cross-amplified in *S. magnicamporum*, eight were polymorphic. At locus 3A, samples of *S. cernua* and *S. parksii* were monomorphic with an allele size of 64. However, *S. vernalis* had an allele size of 61bp for this locus. Locus sp4-1A was also monomorphic across all samples of *S. cernua* and *S. parksii* analyzed, thus these particular loci were not informative and excluded from further analysis. Sp3-11B amplified with three peaks

in *S. cernua* (of) and *S. parksii* and due to difficulties in interpretation, this locus was discarded as well, leaving seven polymorphic loci which were used in the analysis. Locus sp2-12C did not cross amplify in *S. magnicamporum*. Data from the ABI 3100 were scored manually in terms of allele size in base pairs from electropherogram peak traces (Fig 11).

Spiranthes vernalis did not cross amplify with many primers and was not useful for comparative purposes for *S. parksii*. When amplification was successful, some individuals of *S. vernalis* were difficult to interpret because they contained four peaks. These four peak profiles may suggests polyploidy also occur in this species. However, chromosome counts of *S. vernalis* indicate it is a diploid, and polyploidy as well as polyembryony are not known for this species. These peak traces might be a PCR artifact due to non-specific amplification. Regardless, the inclusion of *S. vernalis* did not help clarify relationships within the *S. cernua* complex in this study.

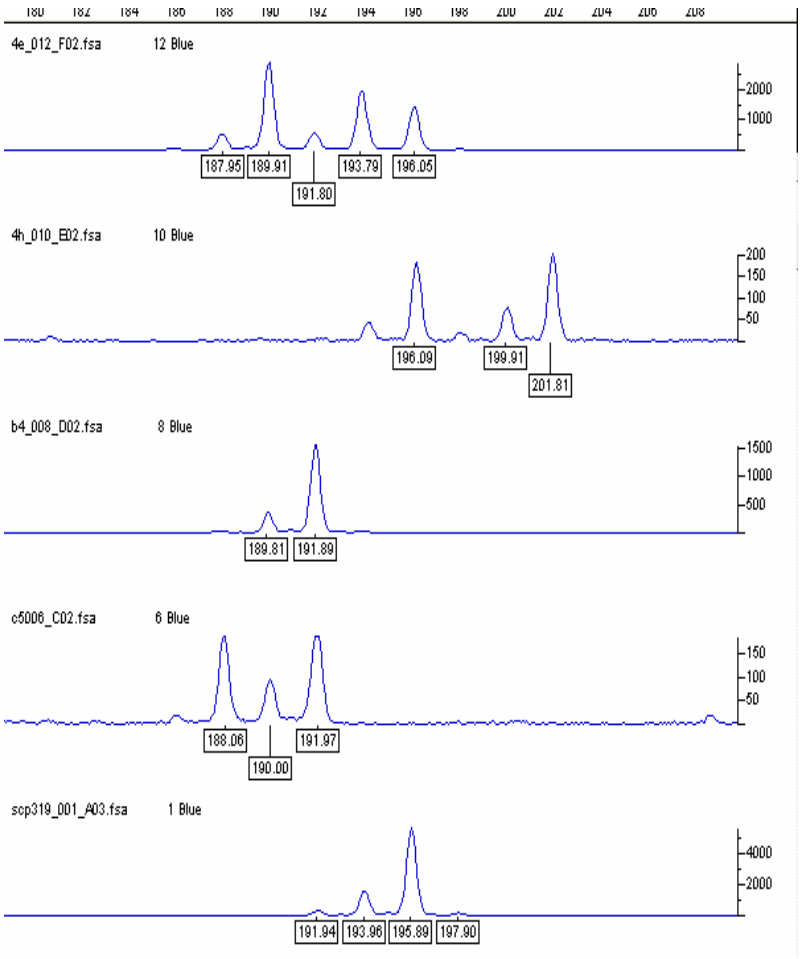

Fig. 11 Electropherogram of microsatellite traces. Individuals of *S. cernua* from eastern locations shown occur in both homozygous (third row) and heterozygous (first and second row) for different alleles at locus sp3-9A. The fifth row represents a homozygous individual of *S. cernua* (cf).

Allelic data were scored manually from peak traces and converted into a binary data matrix in which individuals either had a particular allele and were scored as "1" or did not have an allele for a given locus, and were scored as "0". There were a total of 55 alleles from the seven loci. From these data, average pair wise distances were calculated.

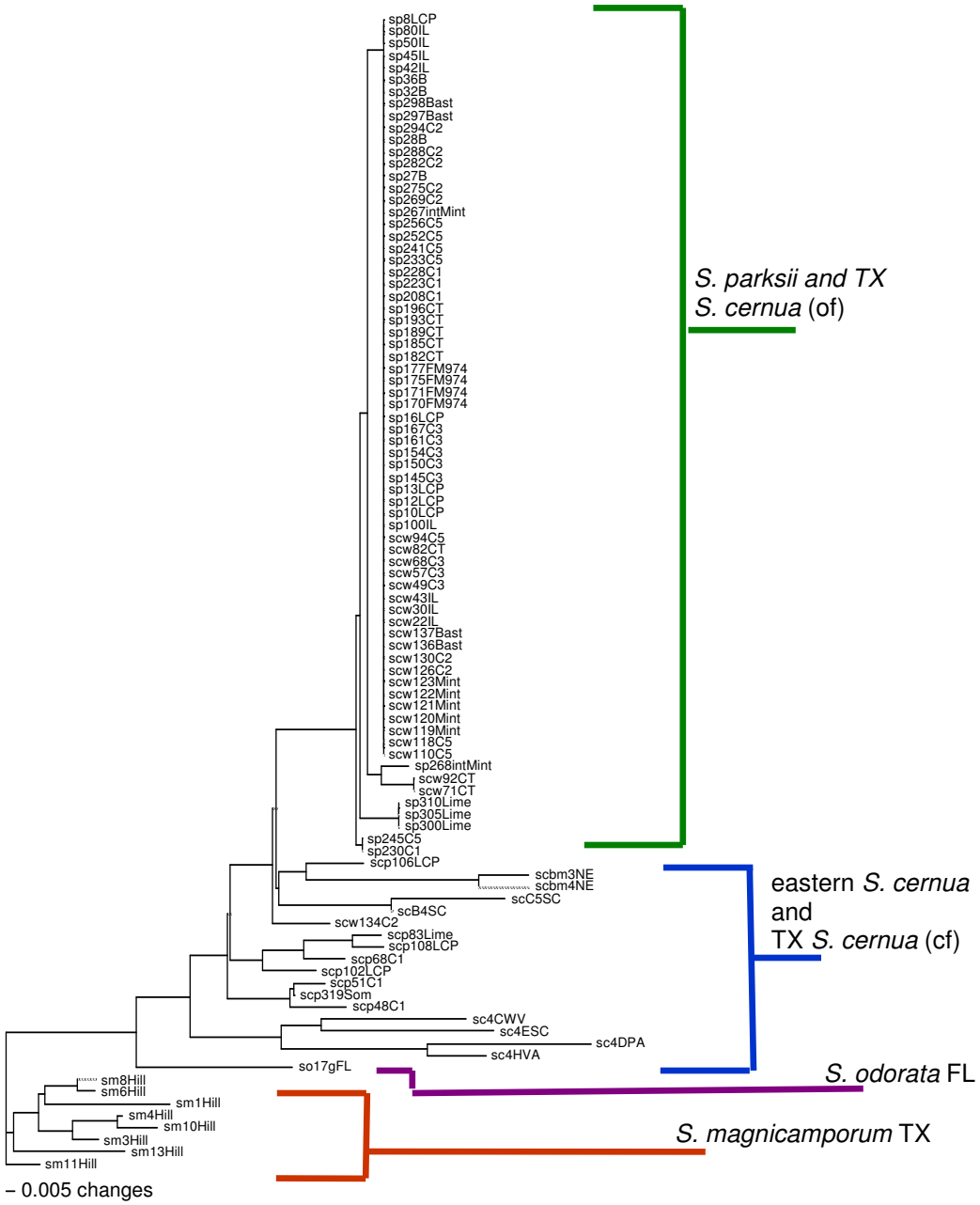

Fig. 12 NJ phylogram constructed from microsatellite data. Population designations are indicated at the end of the sample ID. sm= *S. magnicamporum*; sp= *S. parksii*; scw=*S. cernua* (of); scp= *S. cernua* (cf). so= *S. odorata*. Samples from PA, WV, SC, VA, NE and FL are indicated on the tree.

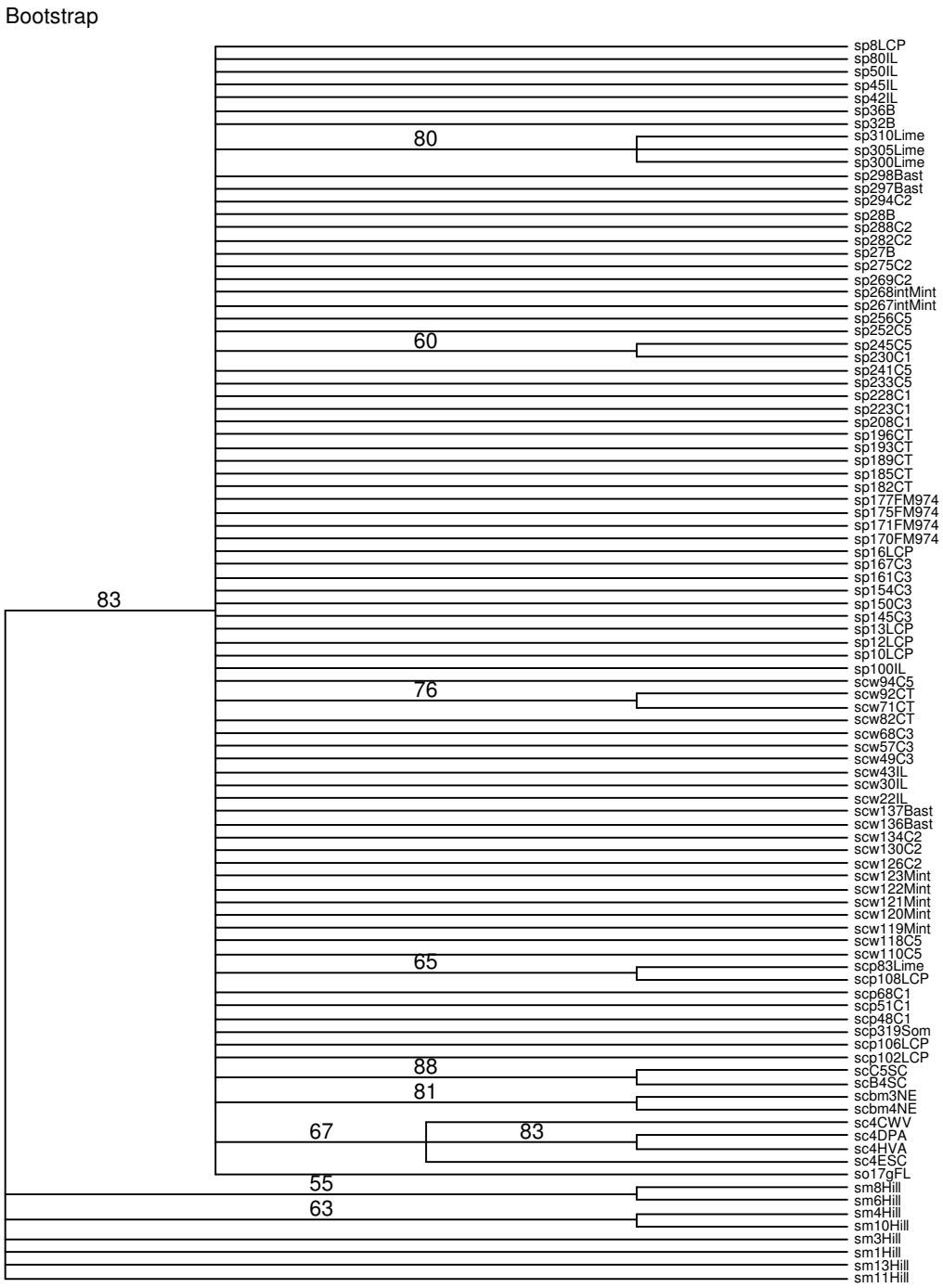

Fig. 13 Bootstrap consensus tree from microsatellite data. Population designations are located at the end of the sample ID. sm= *S. magnicamporum*; sp= *S. parksii*; scw=*S. cernua* (of); scp= *S. cernua* (cf); so = *S. odorata*. Samples from outside of TX are followed by their respective state abbreviations.

Using *S. magnicamporum* as the outgroup, a neighbor joining tree was constructed in PAUP 4.02b (Fig. 12) and a bootstrap analysis was performed with 1,000 replicates (Fig. 13).

Few polymorphisms were detected among samples of *S. parksii* and *S. cernua* (of). Two samples of *S. parksii* and one *S. cernua* (of) (sp245, sp230 and scw134) from three different populations were homozygous at a locus where others were heterozygous. Another individual (sp268int), which was a morphological intermediate between *S. parksii* and *S. cernua* (of) contained an allele also found in *S. cernua* (cf). Two samples of *S. cernua* (of) (scw92 and scw71) from different populations in Brazos county also contained an allele that was also found in *S. cernua* (cf).Finally, three samples of *S. parksii* from Limestone County (sp300, sp305, sp310) contained a unique allele at locus 8D2 not present elsewhere in this dataset.

Individuals from Limestone County grouped together with bootstrap support of 80% due a unique allele at a single locus. Many of the eastern samples of *S. cernua* form groups with strong bootstrap support of 67% and higher. The topology of the tree otherwise is largely unstructured among samples of local *S. cernua* (of) and *S. parksii,* with the exception of a few samples which contain few informative polymorphisms. *Spiranthes parksii* and *S. cernua* (of) are nested within the *S. cernua* group, which also includes *S. cernua* (cf) and *S. cernua* from other states.

Observed heterozygosity was calculated for each taxon across loci (Table 4). Heterozygosity appeared to be fixed in four of the seven loci examined in both *S. parksii* and *S. cernua* (of) (locus sp2-11h, sp2-12c, sp4-9C and 8D2). A majority of these

samples were fixed for alleles 173bp and 177bp at locus sp4-9C. However, two individuals of *S. parksii* and one individual of *S. cernua* (of) were homozygous for allele 173bp. *Spiranthes magnicamporum* contained the lowest H_{obs} with an average value of .32.

The numbers of alleles per locus as well as average number of alleles across loci were calculated for each taxon (Table 5). *S. cernua* contained the highest average number of alleles per locus, while *S. parksii* and *S. cernua* (of) contained the lowest. The value for *S. cernua* (of) may be inflated due to sample "scw134", which contains alleles that other samples of *S. cernua* (of) and *S. parksii* do not and is genetically more similar to *S. cernua* (cf). *S. magnicamporum* as well as *S. cernua* (cf) showed intermediate values for this parameter. These values should be interpreted with caution as *S. magnicamporum* incorporated were from a single population, whereas *S. cernua* from eastern locations were from several, more geographically disjunct populations. Clearly, samples from several populations throughout the range would necessary to obtain better estimates of these two parameters.

Table 4 Observed heterozygosity among taxa. H_{obs} is shown for each locus (columns 3-9) and then averaged across loci (AVE H_{obs}). Taxon (and form if applicable) is indicated on the left followed by samples size (N). NA indicates amplification was not successful.

Taxon	(N)	sp2-11h	sp2-12C	8D2	sp3-3a	sp3-9A	sp4-5E	sp4-9C	AVE H_{obs}
S. parksii	49	1.00	1.00	1.00	0.00	0.00	0.00	0.96	0.57
S. cernua (of)	22	1.00	1.00	1.00	0.00	0.00	0.00	0.95	0.56
S. cernua (cf)	8	0.88	0.38	0.50	0.00	0.25	0.75	0.13	0.41
S. magnicamporum	8	0.25	NA	0.00	0.29	0.38	1.00	0.00	0.32
S. cernua Eastern	10	0.50	0.57	0.50	0.50	0.60	0.60	0.71	0.57

Table 5 Number of alleles per locus. Average number of alleles is indicated by column"A". Taxon (and form if applicable) is indicated on the left followed by samples size (N). NA indicates amplification was not successful.

Taxon	(N)	sp211h	sp212C	8D2	sp3 3a	sp39A	sp45E	sp4 9C	A
S. parksii	49	2.00	2.00	3.00	1.00	2.00	1.00	2.00	1.86
S. cernua (of)	22	2.00	2.00	2.00	2.00	3.00	1.00	2.00	2.00
S. cernua (cf)	8	3.00	4.00	2.00	2.00	4.00	2.00	3.00	2.86
S. magnicamporum	8	3.00	NA	1.00	4.00	2.00	2.00	4.00	2.67
S. cernua Eastern	10	4.00	7.00	3.00	7.00	8.00	3.00	6.00	5.43

Discussion

Breeding system plays a critical role in determining patterns of genetic diversity, and the limited genetic variation detected in *S. parksii* or between S. cernua (of) or S. parksii are likely due to high levels of asexual reproduction. The over-representation of specific genotypes suggests high levels of apomixis occur in both species and is in contrast with the previously held view that local *S. cernua* (of) was likely reproducing sexually with more frequency than *S. parksii*. The occurrence of fixed heterozygous profiles at several microsatellite loci are further support for an asexual reproductive mode and indicative are not suffering from inbreeding depression. The resulting tree (Fig 12) reflects these relationships and shows that *S. parksii* and *S. cernua* (of) together form a polytomy, which occupies much of the tree space.

Four loci (sp2-11H, sp2-21C, 8d2 and sp4-9C) were particularly interesting in that most individuals of *S. cernua* (of) and *S. parksii* contained two identical peaks and showed a fixed heterozygous profile. Individuals at one of these loci,"sp4-9C" (Fig 15), had alleles at 173bp and 177bp. Two explanations for these patterns are possible: The

first is that *S. parksii* and sympatric S. *cernua* (of) are of allopolyploid origin and each contributing parent was homozygous at these loci. If inheritance is disomatic (genomes are divergent enough that only two chromosome pairings are possible during meiosis), these alleles would not segregate into gametes even if sexual reproduction does occur, or they will be perpetuated by the apparently high levels of apomixis. In this scenario, an increase in ploidy level is expected based on the presence of two genomes and seems unlikely based on previous chromosome counts, which indicate no difference in ploidy levels and that both *S. cernua* and *S. parksii* are tetraploid as are most other members of the *S. cernua* complex.

The other possibility is that these individuals show a true fixed heterozygosity due to heterozygous individuals passing their genotype on to progeny through apomixis. This pattern could be broken by recombination events, which would eventually segregate alleles, and result in individuals that are homozygous as well as heterozygous for these particular loci. This is the current favored model for *S. parksii* because three individuals, two *S. parksii* and one *S. cernua* (of) from three populations, were homozygous at this locus for allele 173, indicating that plants are capable of reproducing sexually, though at low frequencies.

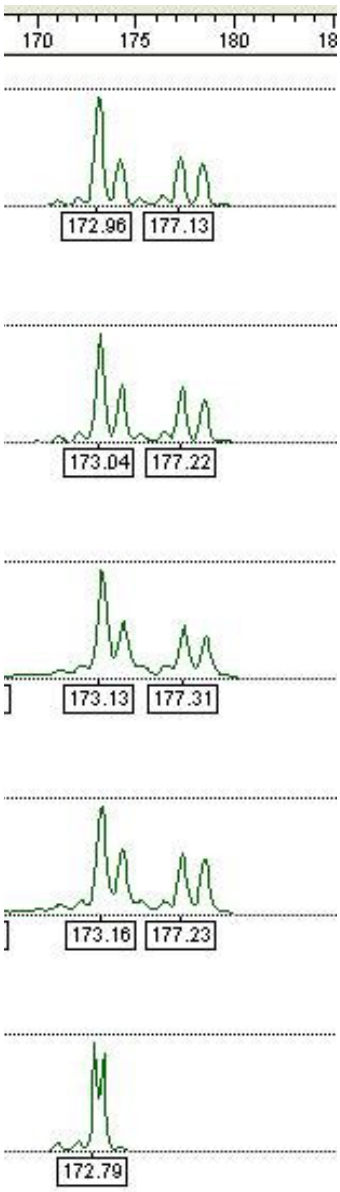

Fig 14. Individuals with a fixed heterozygous profile. Individuals of *S. cernua* (of) (rows one and two) and *S. parksii* (rows three and four) which have fixed heterozygous profiles at locus sp4-9C are shown. The bottom individual is an individual of *S. cernua* (cf) homozygous at the same locus.

Spiranthes cernua (cf) was expected to show low levels of genetic variation compared to either *S. parksii* or *S. cernua* (of). It may seem counterintuitive that this form, recognized largely on the basis of having closed flowers and suspected autogamic or agamospermic reproductive mode, contained more genetic variation than similar taxa with open flowers. These results may be explained by this form's ability to produce open flowers as well as its preference for a more open habitat, providing better access to pollinators compared to individuals with open flowers in a woodland habitat, which is not the preferred habitat for the typical pollinators of *Spiranthes*.

It follows that a morphological discrimination of this form that occurs with open flowers from the open flower, woodland *S. cernua* may be problematic. One particular sample, "scw134", illustrates this quandary as it was originally identified as the open flower form of *S. cernua*, but genetically it grouped with the closed flower samples of *S. cernua* (Fig. 12).Though they may be genetically differentiated, their overlapping habitat as well as similar morphology would make field identification impractical among individuals with open flowers. From this perspective, Sheviak's treatment of the two as forms seems justified.

The higher levels of variation detected in fewer samples examined among this form indicate it does not rely in asexual reproduction to the extent of either *S. parksii* or sympatric, woodland, *S. cernua* (of). There is some evidence that "races" which are intermediate in their reproductive mode, or capable of both apomixis and sexual reproduction, contain up to three embryos per seed but no more (Swamy 1948). An examination of the number of embryos per seed may offer additional support for the idea

that the closed flower form is intermediate in terms of its ability to reproduce sexually as well as asexually. Based on the genetic data, this form is expected to contain fewer embryos per seed than either *S. cernua* (of) or *S. parksii*, both of which appear to be highly clonal and reproducing sexually only on rare occasions. Due to the current lack of understanding of the relationship between polyembryony and asexual reproduction, polyembryony should be used in conjunction with data from other sources (genetic or developmental) as this is an indirect character used to indicate asexual reproduction. However, the correlation between polyembryony and asexual reproduction is probably not strict.

Due to the controversy surrounding the use of microsatellite loci in non-focal taxa, a final note relating to cross amplification of microsatellite loci is warranted. Many caution against using microsatellites in non-focal taxa such as *S. cernua* and *S. magnicamporum* (those more distantly related to the one used to obtain microsatellite sequence data) to avoid ascertainment bias. Ascertainment bias results in an apparent, but biased reduction in genetic diversity due to the fact that regions with longer repeats are more variable and are typically selected for during the marker development phase. Compared to either *S. cernua* (of) or *S. parksii*, *S. magnicamporum*, a diploid taxon, contains more genetic variation in terms of number of alleles per locus (Table 5). It is not clear if ascertainment bias is affecting these results. The higher levels of genotypic as well as allelic diversity found in *S. magnicamporum* are unusual, particularly considering samples used in the analysis were from a single population. This result is likely a consequence of its sexual reproductive mode. *Spiranthes magnicamporum*, a

diploid relative of *S. parksii*, did show slightly lower levels of average H_{obs} across loci (Table 4), which is consistent with results from other studies that show diploid species commonly harbor less heterozygosity than their polyploid relatives.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

All three techniques employed in this study, chloroplast sequence from the non-coding trnT-trnF region, AFLPs and microsatellite markers were congruent in that they detected little or no genetic variation in *S. parksii* and the sympatric open flower (or woodland) form of *S. cernua.* Results from this study offer support for a predominantly asexual mode of reproduction with the capability of occasional sexual reproduction. The markers did detect genetic differentiation of as well as genetic variation in the closed flower (or peloric) form of *S. cernua*, which suggest higher levels of sexual reproduction may occur in this group. Finally, the alleles present in both *S. parksii* and sympatric *S. cernua* (of) are also found in the more widely distributed closed flower form of *S. cernua* indicating their origin(s) are from this group. These data suggest *S. parksii* and sympatric *S. cernua* (of) share a very recent common ancestor.

Data from the microsatellite loci revealed the most detail concerning the breeding system of *S. parksii* as well as genetic relationships within the *S. cernua* complex. These markers have the potential to further our understanding of the relationships within the *S. cernua* complex including *S. parksii*.

Origins of *S. parksii*

There has been much speculation on the origins of *S. parksii*, ranging from interspecific hybridization to founder events. However, there is no evidence that *S. parksii* contains

alleles that are unique to any two potential progenitors. Also, though a founder event would result in a similar lack of genetic variation, it would result in the more widespread, sympatric *S. cernua* showing much higher levels of diversity than its putative derivative, *S. parksii*. The founder hypothesis is not consistent with the observation of fixed heterozygosity at many of the microsatellite loci or with the similar average H_{obs} among the two (Table 4). Another explanation for the lack of diversity between the two species is that *S. parksii* and *S. cernua* (of) are recently derived species and have evolved few genetic differences, which have thus far gone undetected. A third possibility is that *S. parksii* as well as sympatric *S. cernua* (of) are not genetically differentiated and morphological differences between them are due strictly to environmental or developmental reasons.

Though specific details of its origin (or origins) are still unknown, it seems that *S. parksii* and *S. cernua* (of) are both derivatives of the more widely distributed *S. cernua* complex. Because *S. parksii* and sympatric *S. cernua* (of) share alleles and contain less allelic variation than either *S. cernua* (cf) or *S. cernua* from other locations indicates they are likely very closely related.

These two taxa occur along the edge of the distribution of *S. cernua*. The apparent high level of apomixis in them may be an example of an adaptation to limited pollinator recruitment, which is often encountered along the periphery of a population. This idea has some anecdotal support from those who monitor populations reporting little to no pollinator activity in *S. parksii*. Yet, others who have studied *S. cernua* in other geographic areas report many pollinators visiting flowers, which suggest a sexual

reproductive mode may be more common for *S. cernua* from these other locations. A follow up on sexual populations of the complex from other geographic locations might provide additional support for this theory as populations with observed pollinator activity would be expected to harbor much higher levels of genetic variation. A previous study detected isozyme variation within populations of *S. cernua* in Nebraska suggesting sexual reproduction is more common among individuals from this particular location (Unpublished, J. Schmidt 1992).

Alternatively, *Spiranthes* species occurring in woodland habitat may be specifically adapted to their habitat, which naturally has a low occurrence of potential pollinators, due to unique combinations of genes from sexual *S. cernua.* Asexual reproduction is possibly selected for in a woodland habitat for this reason and if reproductive mode is an expression of genetic rather than environmental factors, an increase in pollinators will not increase sexual reproduction.

There is some speculation that polyploid, asexual species accumulate “mutational junk” due to the buffering capacity conferred from the presence of an additional genome. It is feasible that sexual reproduction becomes selected against due to the potentially lethal alleles occurring in a homozygous state in sexually produced offspring. Thus, the asexual reproductive mode and polyploidy become selected for as well as tightly linked. However, empirical evidence to support this is lacking. The genetic controls of apomixis for this genus are a black box and without more detailed genetic and developmental studies, the amount of plasticity in the reproductive mode of *Spiranthes* will remain unknown.

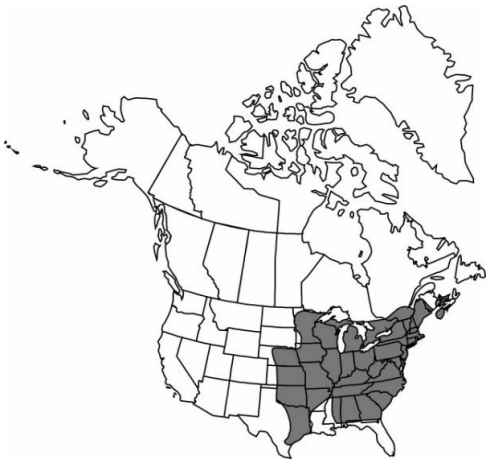

Fig. 15. Distribution of *Spiranthes cernua*. (Flora of North America Editorial Committee, eds. 1993+).

Spiranthes parksii and species concepts

Organisms with mixed mating systems often result in confusing taxonomies and caution must be used when assigning specific rank to members of the *S. cernua* complex due to a potentially high number of local varieties or races which vary in morphology and reproductive mode. It is important to emphasize that species circumscription are largely and necessarily (due to practical reasons) based on morphological comparisons, yet are lacking in set standardization due to extreme variation in biological diversity. Application of hierarchical categories to the biological diversity of *Spiranthes* is a daunting task, yet it must be done when specific rank is of legal significance. Several concepts are used to define species, none of which are universally accepted. Still it is useful to consider each concept as it relates to the issue of species status of *S. parksii*.

Because of the prevalence of an asexual reproductive mode, the commonly accepted biological species concept does not apply because it recognizes species as

groups of actual or potential interbreeding organisms isolated from other such organisms. Individuals of *S. parksii* are likely not interbreeding with other individuals, yet they could be considered reproductively isolated as well. The converse of this concept is the recognition species concept which recognizes species are groups of organisms sharing a common fertilization system. Again, in clonal individuals that do not rely on fertilization to produce offspring, this concept does not apply.

The agamospecies concept defines a species as a lineage descending from a uniparental organism and has been applied to members of the genus *Rubus* and *Taraxacum* both of which are notorious for their apomictic modes of reproduction. This species concept may at first seem to apply; however, that *S. cernua* and *S. parksii* are thus far genetically indistinguishable implies they may be a single lineage and constitute the same species. Additional problems with the concept arise when an individual of the species (for example a local individual of *S. cernua*) from which the agamospecies arose is more related to the agamospecies (*S. parksii*) than to other members its same species (*S. cernua* from other locations). Approaching species concepts in this manner could mean local *S. cernua* as well as *S. parksii* constitute a single species separate from individuals of *S. cernua* from other locations.

The ecological species concept defines species as groups of similar organisms occupying a similar niche in space and time. If a species is defined as being adapted to a particular set of resources, *S. parksii* could not be considered a species due to the fact it is often sympatric with *S. cernua* and the two share similar resources. More specific

habitat requirements have been described for *S. parksii*, however, plants of both morphologies are often found within inches of each other.

Under the phylogenetic species concept, the majority of *S. parksii* would not be a species distinct from sympatric *S. cernua*, however, plants in Limestone county which differ at locus 8D2 by having allele sizes of 92bp and 96bp rather than 92bp and 94bp as all other individuals of *S. parksii* may be considered a separate species because this concept defines species as assemblages of individuals which are diagnosably different from other such assemblages. Also under this concept, *S. parksii* and sympatric *S. cernua* could be considered a new and distinct species from allopatric *S. cernua* because they are diagnosably different. Interpreting this species concept strictly would yield an unreasonable number of species in a widespread species complex such as *S. cernua* and the use of this concept would depend on molecular markers to diagnose differences, making this species concept highly impractical for this particular group of plants.

Under the morphological species concept, groups which share a unique set of morphological characters are considered species. Thus far, this is the concept which best applies to *S. parksii* because *S. parksii* appears to be contain morphologically distinct characters which separate it from *S. cernua* (of), though there is much variation and intermediate forms can be found. The major drawback to this species concept is that the underlying cause of the morphological variation in *S. parksii* is not well understood and based on these results cannot be correlated with genetic factors. Additionally, some of the unique morphology seen within *S. parksii* is not unique within the complex. For example, white-tipped bracts also occur in *S. casei* Catling and Cruise, another suspected

apomictic species which occurs in the northern United States and southern Canada, and is thought to be derived from *S. cernua*. A carefully planned common garden experiment (in which seeds collected should be from bagged and emasculated inflorescences to exclude any possibility of sexually produced seeds) would inform us as to whether these morphological characters are heritable or an expression of environmental influence. Because identification of this species is dependent on a small set of variable morphological characters, morphological studies are of particular interest.

The cladistic species concept claims each new branch point on a phylogenetic tree represents a speciation event. From this perspective, branching points do not clearly separate sympatric *S. cernua* and *S. parksii*, thus they would not be considered unique species based on these data. However, if the tree were to be constructed with morphological data, there may be a clear branching point between the two. A detailed and systematic morphological study would need to be done to determine whether or not *S. parksii* and sympatric *S. cernua* cluster into two significantly different groups. The dilemma created by recognizing *S. parksii*, is that *S. cernua* becomes a parayphyletic assemblage due to the exclusion of its descendents. If species are to reflect natural, monophyletic groups, lineages such as *S. parksii* and local *S. cernua* (of) must be included with *S. cernua*. However, recognition of *S. cernua* and all descendents may result in a single polyphyletic species due to it suspected multiple origins from the related diploid species, *S. magnicamporum*, *S. ochroleuca* (Rydb.) Rydb. and *S. odorata* (Nutt.) Lindl., which are thought to be capable of donating their genes into *S. cernua* through hybridization (Sheviak, 1982). Either treatment is not satisfactory from a

cladistic perspective. While maintaining monophyly in species is certainly a desirable goal, it may be impractical for this group due to the inordinate amount of knowledge required about branching patterns and relationships within *S. cernua* and among related species.

Ultimately, these taxonomic problems are centered around difficulties in the circumscription of *S. cernua*, a group with potentially multiple hybrid origins, variation in chromosome number and structure, reproductive mode, morphology as well as habitat preference. While this genetic study enhances our understanding of local *Spiranthes* in east-central Texas, it adds a small piece to a large and highly complicated puzzle. It is possible that other asexual lineages derived from it will be described. These lineages may also have restricted distributions, whether due to recent origins, unique genetic combinations allowing adaptations to specific habitats, or any number of stochastic processes. Regardless of the taxonomic treatment of *S. parksii*, this will certainly not be the last time *Spiranthes* and species concepts are discussed.

REFERENCES

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman and Hall, New York.

Bellstedt DU, Linder HP, Harley EH (2001) Phylogenetic relationships in *Disa* based on non-coding TrnL-trnF chloroplast sequences: evidence of numerous repeat regions. *American Journal of Botany*, **88**, 2088-2100.

Bridges EL, Orzell SL (1989a) Additions and noteworthy vascular plant collections from Texas and Louisiana, with historical, ecological, and geographical notes. *Phytologia,* **66**, 12-69.

Catling PM, McIntosh KL (1979) Rediscovery of *Spiranthes parksii*. *Sida,* **8**,188-193.

Catling PM (1982) Breeding systems of North American *Spiranthes* (Orchidaceae). *Canadian Journal of Botany*, **60**, 3017-3039.

Catling PM (1983) Pollination of Northeastern North American *Spiranthes* (Orchidaceae) *Canadian Journal of Botany,* **61**, 1080-1093.

Correll DS (1950) *Native Orchids of N. America North of Mexico*. Chronica Botanica Co., Waltham, Massachusetts.

Cronn RC, Small RL, Haselkorn T, Wendel JF (2002) Rapid diversification of the cotton genus (Gossypium: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. *American Journal of Botany,* **89**(4), 707-725.

Dietrich WF, Miller J, Steen R, Merchant MA, Damron-Boles D, *et al* (1996) A comprehensive genetic map of the mouse genome. *Nature*, **380**, 149-152.

Forrest AD, Hollingsworth ML, Hollingsworth PM, Sydes C, Bateman RM (2004) Population genetic structure in European populations of *Spiranthes romanzoffiana* set in the context of other genetic studies on orchids. *Heredity*, **92**(3), 218-27.

Harris H (1966) Enzyme polymorphism in man. *Proceedings of the Royal Society London Series B* **164**, 298-310.

Leavitt RG (1901) Notes on the embryology of some New England orchids. *Rhodora,* **3**, 202-205.

Lewontin RC, Hubby JH (1966) A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of

heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics,* **54**, 595-609.

Pepper AE, Norwood LE (2001) Evolution of *Caulanthus amplexicaulus* var *barbarae* (Brassicaceae), a rare serpentine endemic plant: a molecular phylogenetic perspective. *American Journal of Botany,* **88**, 1479-1489.

Salazar GA, Chase MW, Soto MA, Ingrouille, M (2003) Phylogenetics of Cranichideae with emphasis on Spiranthinae (Orchidaceae, Orchidoideae): evidence from plastid and nuclear DNA sequences. *American Journal of Botany,* **90**, 777-795.

Schmidt JM, Antlfinger AE (1992) The level of agamospermy in a Nebraska population of *Spiranthes cernua* (Orchidaceae). *American Journal of Botany* **79**, 501-507.

Sheviak C (1982) Biosystematic Study of the *Spriranthes cernua* Complex. New York State Museum. Bulletin No. 448.

Sheviak CJ, Brown, PM (2003) *Spiranthes*. In.: *Flora of North America North of Mexico* (ed. Flora of North America Editorial Committee), vol. 26, p. 540. Oxford University Press, New York.

Swamy BGL, (1948) Agamospermy in *Spiranthes cernua*. *Lloydia*, **11**, 149-162.

Taberlet P, Gielly, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, **17**, 1105 1109.

Trapnell DW, Hamrick, JL (2004) Partitioning nuclear and chloroplast variation at multiple spatial scales in the neotropical epiphytic orchid, *Laelia rubescens*. *Molecular Ecology*, **13**, 2655-2666.

Texas A&M Bioinformatics Working Group.
http://www.csdl.tamu.edu/FLORA/tamuherb.htm (Map retrieved on 10/12/05).

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, *et al* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research,* **23**, 4407.

Wang Z, Weber JL, Zhong G, Tanksley SD (1994) Survey of plant short tandem DNA repeats. *Theoeticalr Applied Genetics,* **88**,1–6.

VITA

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